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Amplification of mixed lineage leukemia gene perturbs hematopoiesis and cooperates with partial tandem duplication to induce acute myeloid leukemia

Genetic alternation of the mixed lineage leukemia (*MLL*) gene can be found in up to 10% of acute myeloid leukemia (AML).^{1,2} Similar to *MLL* fusions and *MLL* par-

tial tandem duplication (PTD), *MLL* amplification (*MLL*(n)) is reported in approximately 1% AML and myelodysplastic syndrome (MDS) associates with adverse treatment outcomes.^{3,4} In spite of the success in modeling AML induced by *MLL* fusions,¹ there has been no *in vivo* disease model for *MLL*(n) leukemia. On the other hand, while previous studies have proposed a recessive gain-of-function by *MLL*-PTD in suppressing the expression of wild-type *MLL* in normal karyotype

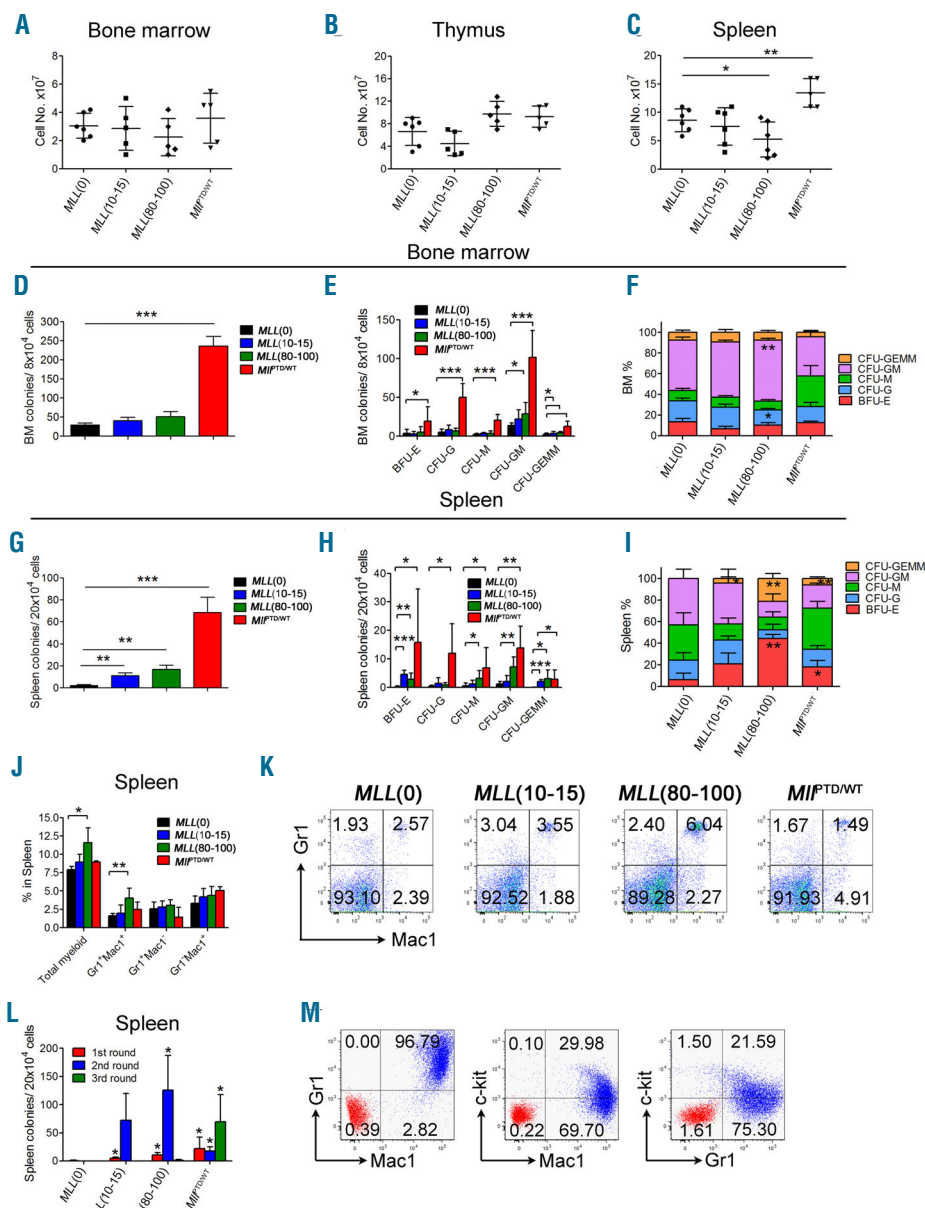


Figure 1. *MLL* amplification perturbs myeloid development in mouse models. Total cell counts in (A) bone marrow (2 femurs), (B) thymus and (C) spleen of the control *MLL*(0), *MLL*(10-15), *MLL*(80-100) and *MLL*^{PTD/WT} mice. (D) Total colony numbers generated from bone marrow cells of *MLL*(n) and *MLL*^{PTD/WT} mice. Bone marrow colonies were classified into burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte/macrophage (CFU-GM), and CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM). (E) Absolute number and (F) relative proportion of each colony type from the bone marrow of indicated genotypes. (G) Total colony numbers formed from splenocytes of *MLL*(0) control, *MLL*(n) and *MLL*^{PTD/WT} mice. (H) Absolute number and (I) relative proportion of each colony type from the spleen of indicated genotypes. (J) Percentages of myeloid cell populations (Total: Gr1⁺ or Mac1⁺, Gr1⁺Mac1⁺, Gr1⁺Mac1⁻ and Gr1⁻Mac1⁺) in spleens of *MLL*(0) control, *MLL*(n) and *MLL*^{PTD/WT} mice. (K) Representative flow cytometry plots showing percentages of Gr1⁺Mac1⁺ cell population in spleens of *MLL*(0) control, *MLL*(n) and *MLL*^{PTD/WT} mice. (L) Splenocytes (20x10⁴ cells) from different mice were seeded into methylcellulose for replating assays. Enumeration of colonies and replating of cells to the next round were performed after 6 days of culture. (M) Flow cytometry analysis of the third round of replating *MLL*^{PTD/WT} splenic colonies. Red dots, unstained cells; blue dots, cells stained with indicated antibodies. Results in panel A to C were obtained from 5 independent experiments, panel D to I were obtained from 6 independent experiments, whereas panel J and L were obtained from 5 independent experiments. Bar graphs show mean+S.E.M. (**P*<0.05, ***P*<0.01 and ****P*<0.001, paired t-test).

AML,⁵ both $Mll^{PTD/WT}$ and $Mll^{PTD/-}$ mice failed to develop leukemia, thus questioning the role of *MLL*-PTD in acute leukemogenesis and indicating a requirement of cooperating mutation(s) for full malignant transformation.^{6,7} Intriguingly, *MLL*-PTD can be found to co-exist with *MLL*(n) in AML patients,^{8,9} suggesting an alternative pathological relationship between these 2 *MLL* aberrations in myeloid leukemogenesis.

To gain insights into the role of *MLL*(n) and *MLL*-PTD in hematopoietic development, we generated an *in vivo* mouse model of *MLL* amplification where the full-length human *MLL* cDNA was expressed under the murine *Scl*-promoter/3'enhancer (Online Supplementary Figure S1A), which has been successfully used to drive oncogene expression in hematopoietic stem and progenitor

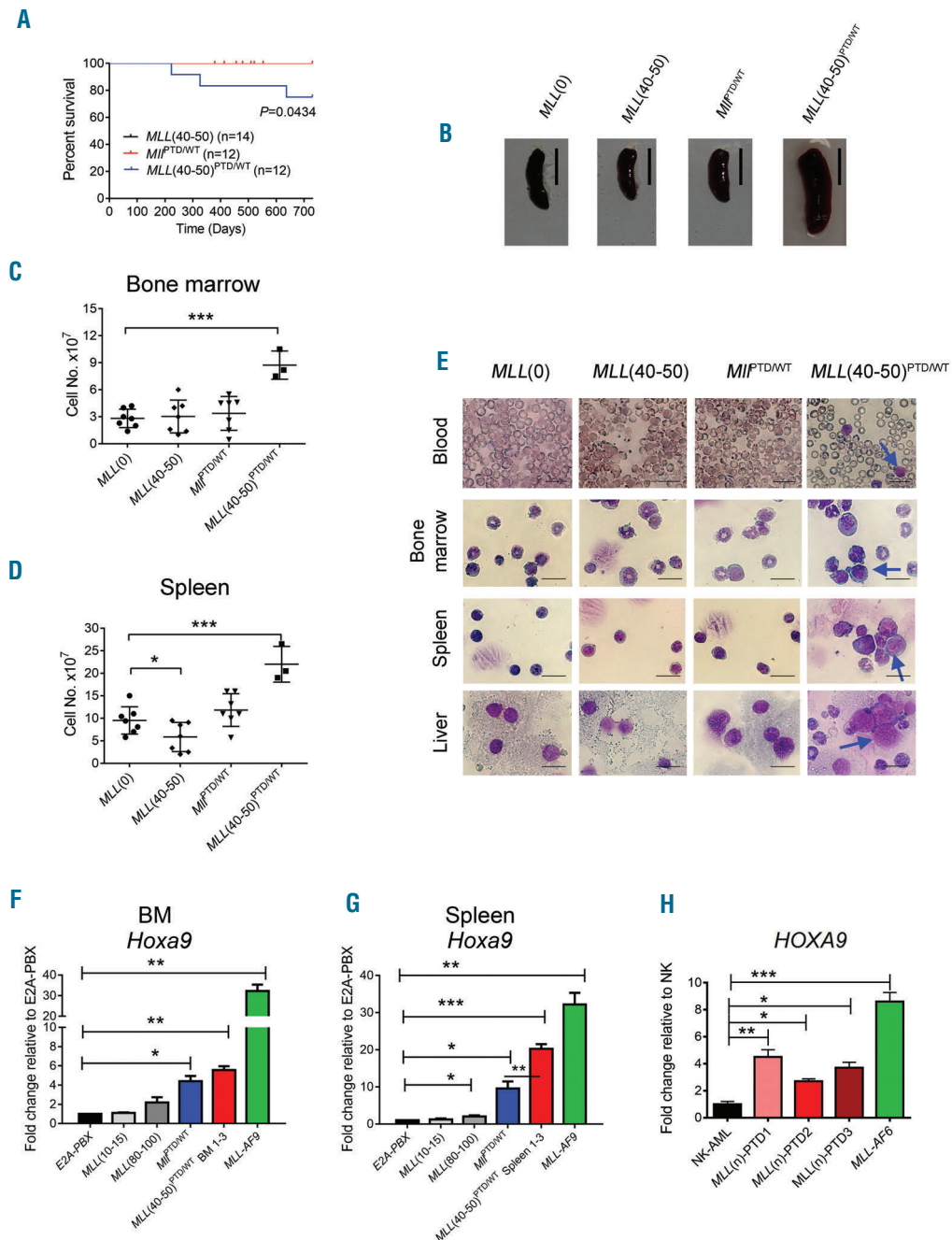


Figure 2. Potential cooperation between *MLL* amplification and *MLL*-PTD results in leukemic transformation in $MLL(40-50)^{PTD/WT}$ mice. (A) Survival curves of $MLL(40-50)$ (n=14), $Mll^{PTD/WT}$ (n=12) and $MLL(40-50)^{PTD/WT}$ (n=12) mice. Three out of 12 $MLL(40-50)^{PTD/WT}$ mice developed AML but none of the control mice showed any sign of disease (* $P < 0.05$). (B) Representative images of spleens from $MLL(0)$, $MLL(40-50)$, $Mll^{PTD/WT}$ and leukemic $MLL(40-50)^{PTD/WT}$ mice. All control mice were age-matched to leukemic mice. Scale bar, 1 cm. Total cell counts in (C) bone marrow (2 femurs) and (D) spleens of $MLL(0)$, $MLL(80-100)$, $Mll^{PTD/WT}$ and leukemic $MLL(40-50)^{PTD/WT}$ mice. (E) Peripheral blood smears and cytopins of the cells prepared from the bone marrow, spleen and liver of $MLL(0)$, $MLL(40-50)$, $Mll^{PTD/WT}$ and $MLL(40-50)^{PTD/WT}$ mice are shown. Leukemic blasts are indicated by blue arrows. Cells were stained with May-Grunwald-Giemsa. Scale bars represent 30 μ m. (F) Relative expression levels of *Hoxa9* in MLL (n), $Mll^{PTD/WT}$, leukemic $MLL(40-50)^{PTD/WT}$ bone marrow and MLL -AF9 leukemic cells compared to $E2A$ -PBX cells by q-RT-PCR. (G) Relative expression levels of *Hoxa9* in MLL (n), $Mll^{PTD/WT}$, leukemic $MLL(40-50)^{PTD/WT}$ splenocytes and MLL -AF9 leukemic cells compared to $E2A$ -PBX leukemic cells by q-RT-PCR. (H) Relative expression of *HOXA9* in human MLL (n)-PTD bone marrow cells and in normal karyotype AML (NK-AML) and MLL -AF6 expressing human bone marrow cells. Bar graphs show mean \pm S.E.M. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, paired t-test).

cells (HSPCs) resulting in AML.¹⁰ Two different founders with 10-15 and 40-50 copies of concatemeric transgenes were selected for establishment of stable lines containing 10-15 (*MLL*(10-15)) or 80-100 copies (*MLL*(80-100)) of *MLL*, respectively (Online Supplementary Methods and Online Supplementary Figure S1B,C). Western blotting and quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed the transgene expression as being, approximately, a 3-5- and 6-10-fold increase in the bone marrow and spleen of *MLL*(10-15) and *MLL*(80-100) mice, respectively (Online Supplementary Figure S2A,B). These findings are consistent with previously reported *Scl*-enhancer's property¹⁰ and in line with the expression level of *MLL* in human MDS/AML patients carrying 5-10 or more than 10 copies of *MLL* with, approximately, a 1.5- and 9-fold increase, respectively.¹¹

To investigate the impacts of *MLL* amplification on normal hematopoiesis, *MLL*(n) mice at three months old were analyzed in parallel with *MLl*^{FTD/WT} mice and non-transgenic control (*MLL*(0)). Although no significant difference in total cellularity was found in bone marrow and thymus, a significant increase in splenic cells was observed in *MLl*^{FTD/WT} mice (Figure 1A-C). In contrast, a reduction in splenic cells was found in *MLL*(n) mice, especially in *MLL*(80-100) mice (Figure 1C), suggestive of a mild differentiation and/or proliferation defect. Consistently, the splenocytes of *MLL*(10-15) and *MLL*(80-100) mice displayed a trend towards a significant increase in the percentage of Annexin-V⁺PI⁻ apoptotic cells compared to those of *MLL*(0) mice (Online Supplementary Figure S2C), providing a potential explanation for the lower cellularity observed in the spleen of *MLL*(n) mice. Functionally, *MLl*^{FTD/WT} bone marrows produced approximately 4 times more colonies than those by *MLL*(n) and the control (Figure 1D,E). While the ratio between each colony type remained constant in *MLl*^{FTD/WT}, *MLL*(80-100) had a significant increase in the percentage of colony forming units-granulocyte/monocyte (CFU-GM) and a decrease in the percentage of colony forming unit-granulocyte (CFU-G; Figure 1F), consistent with a mild shift to immature myeloid. *MLL*(n) splenocytes showed a slightly but significantly higher colony forming ability (Figure 1G,H), where they consistently exhibited an increase in immature colony forming units-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) and burst forming units-erythroid (BFU-E; Figure 1I). The degrees of expansion were even more notable in *MLL*(n) mice with a higher copy number of the *MLL* transgene (Figure 1G-I), suggesting an aberrant selection of proliferative myeloid clones. While there was no apparent difference in the proportions of B cells and T cells in these mice (Online Supplementary Figure S3A), an expansion of the Gr-1⁺Mac-1⁺ cell population was found in the *MLL*(80-100) spleen (Figure 1J,K). Consistently, a transient enhanced replating ability was observed in *MLL*(n) and *MLl*^{FTD/WT} splenocytes (Figure 1L). Compared to non-transgenic controls, *MLL*(10-15) and *MLL*(80-100) splenocytes, the latter in particular, were able to form a substantial number of secondary colonies (Figure 1L). *MLl*^{FTD/WT} splenocytes were able to generate compact myeloid colonies even in the third round of replating (Figure 1L,M). In contrast, there was no obvious difference in the replating ability of bone marrow cells (Online Supplementary Figure S3B), consistent with the observed phenotypes in these mice (Figure 1A-C).

Apart from a significant decrease in the percentage of common lymphoid progenitors (CLPs) in the bone mar-

row of *MLl*^{FTD/WT} mice, further investigation into the effect of *MLL*(n) and PTD on early HSPC populations revealed no significant difference in hematopoietic stem cells (long-term (LT)-HSCs, short-term (ST)-HSCs), early myeloid/lymphoid progenitor (multipotent progenitors [MPPs], common myeloid progenitors [CMPs], granulocyte-monocyte progenitors [GMPs], megakaryocyte-erythroid progenitors [MEPs]), or lymphoid (B-[B220⁺], T-[CD4⁺CD8⁻ and CD4⁺CD8⁺]) cell populations in their bone marrows (Online Supplementary Figure S3C-E). RT-qPCR confirmed the over-expression of *MLL* in c-kit⁺ HSPC bone marrow cells, albeit to a lesser extent than in whole bone marrow cells in *MLL*(n) mice (Online Supplementary Figure S3F,G), suggesting that mild over-expression of *MLL* or *MLL*-PTD alone has a rather limited impact on HSPC compartments. Consistently, none of the animals developed leukemia during two years of observation (Online Supplementary Figure S3H), indicating that additional events are required for overt leukemia as observed in humans.

On the other hand, the recurrent reports of AML possessing both *MLL*(n) and *MLL*-PTD^{8,9} raise the intriguing possibility that these 2 different *MLL* aberrations may collaborate in acute leukemogenesis.¹ To address this long outstanding question, we crossed *MLL*(80-100) with *MLl*^{FTD/WT} mice to produce a novel double mutant *MLL*(40-50)^{FTD/WT} mouse (Online Supplementary Figure S1C and S4). In contrast to *MLL*(40-50) and *MLl*^{FTD/WT} controls, which did not develop any malignancy, 3 out of 12 *MLL*(40-50)^{FTD/WT} mice succumbed to AML with immature myeloblasts and splenomegaly after 7 to 21 months ($P=0.0434$) (Figure 2A-D). Examination of peripheral blood revealed anemia and circulating myeloblasts (Figure 2E), which also infiltrated the spleen and liver and resulted in an alteration in their normal architecture (Online Supplementary Figure S5 and Online Supplementary Table S1). Flow cytometric analysis further confirmed expansion of Gr-1⁺Mac-1⁺c-kitlow myeloblasts (Online Supplementary Figure S6A), which was in contrast to a drastic reduction of B220⁺ cells (Online Supplementary Figure S6B). These results indicate that *MLL* amplification and *MLL*-PTD cooperate to block normal myeloid differentiation and to induce their aberrant expansion leading to overt AML.

To gain further molecular insights underlying these cooperating *MLL* aberrations, we assessed the transcriptional and epigenetic status of *Hoxa9*, a key downstream target for *MLL* leukemogenesis.^{12,13} By comparison with *MLL*(40-50), *MLl*^{FTD/WT} controls and *E2A-PBX* mouse leukemic cells, which expressed basal level of *Hoxa9*, *MLL*(40-50)^{FTD/WT} leukemic cells, in particular those from the spleen, expressed significant levels of *Hoxa9*; almost 10-fold higher than *MLL*(40-50) and twice more than *MLl*^{FTD/WT}, showing the possibility of functional cooperation in *MLL* aberrations (Figure 2F-G). In addition to *Hoxa9*, a considerably higher level of *Meis1*,¹ another target for *MLL* leukemogenesis, was also detected in *MLL*(80-100) and *MLL*(40-50)^{FTD/WT} bone marrow cells, which provides an explanation for the transformation in the double mutants (Online Supplementary Figure S7A). To further confirm this finding in the relevant human disease, we also examined the expression and epigenetic status of *HOXA9* in primary human AML blasts carrying both *MLL*(n) and *MLL*-PTD (Online Supplementary Figure S7B). *HOXA9* was significantly over-expressed in all 3 *MLL*(n)-PTD samples and *MLL*-AF6 leukemia as compared to normal karyotype AML (Figure 2H).

At the epigenetic level, similar to *MLL*-AF9 mouse leukemic cells, we observed an upregulation of the H3K4me3 and H3K79me2 activation marks and a reduction of H3K9me2 repressive mark in *MLL*(40-50)^{PTD/WT} bone marrow cells, where higher levels of *MLL* expression were detected in comparison with non-transgenic controls or *MLL*-PTD (Figure 3A,B). Interestingly, Kühn *et al.* have observed profound enrichment of H3K79me2 marks at the highly expressed *HOXA* cluster locus in *MLL*-PTD positive leukemia cell lines.¹⁴ They found that DOT1L inhibition abrogated H3K79me2 and led to downregulation of *MLL* targets in *MLL*-PTD leukemia cell lines,¹⁴ suggesting that DOT1L is a potential therapeutic target for *MLL*-PTD leukemia. Consistent with the mouse model, we also observed similar histone modifica-

tion patterns in human *MLL*(n)-PTD blasts (Figure 3C). While *MLL*(n)-PTD samples exhibited an increased H3K79me2 mark, it was significantly lower than that seen in *MLL* fusion leukemia. Conversely, H3K4me3 was invariably present at a high level in all *MLL*(n)-PTD samples (Figure 3C), which is in line with the detection of elevated expression of wild-type *MLL* in primary AML patients (Figure 3D), suggesting that H3K4me3 mediated by the *MLL*/*SET* family may also play a critical role for activation of downstream targets in these leukemias. To further test this hypothesis, pharmacological inhibition of *MLL* catalytic activity by a small molecule inhibitor WDR-010315 significantly reduced cell viability (Figure 3E), induced cell differentiation (Figure 3F) and repressed ribonucleic acid (RNA) expression of *Meis1* (Figure 3G) of

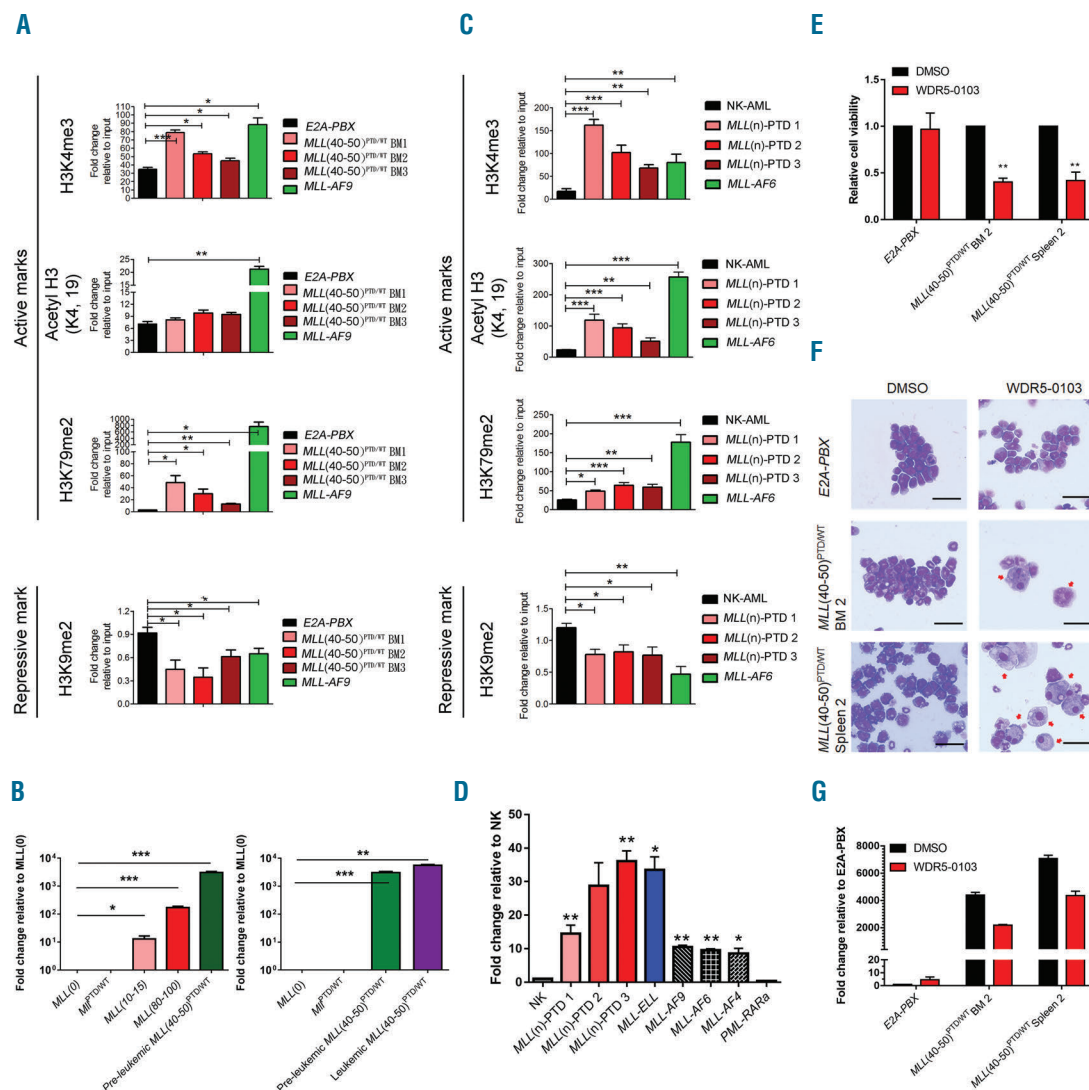


Figure 3. RNA expression and histone modifications of *HOXA9* in *MLL*(40-50)^{PTD/WT} mice and *MLL*(n)-PTD patients. (A) Fold change of H3K4me3, acetyl H3, H3K79me2 and H3K9me2 histone marks within the *Hoxa9* locus are shown. Results were obtained from 4 independent experiments. (B) Relative expression levels of *MLL* in the bone marrow of *MLL*(10-15), *MLL*(n), and pre-leukemic *MLL*(40-50)^{PTD/WT} mice compared to age-matched wild-type *MLL*(0) mice (left panel), pre-leukemic and leukemic *MLL*(40-50)^{PTD/WT} mice compared to age-matched wild-type *MLL*(0) mice (right panel). Results were obtained from 5 mice with the exception of *MLL*(40-50)^{PTD/WT} where only 3 mice were available. (C) Fold change of H3K4me3, acetyl H3, H3K79me2 and H3K9me2 histone marks within the *HOXA9* locus using bone marrow cells from human *MLL*(n)-PTD, NK-AML, and *MLL*-AF6, and *MLL*-AF6 samples. Results were obtained from 3 independent experiments. (D) Relative expression levels of *MLL* in human primary samples with *MLL*(n)-PTDs, *MLL* fusions and *PML-RARα* compared to NK-AML. Expression of *Gapdh* was used as an endogenous control to standardize the cDNAs. Results were obtained from 3 independent experiments. Bar graphs show mean±S.E.M. (**P*<0.05, ***P*<0.01 and ****P*<0.001, paired *t*-test). (E) Relative cell viability (*n*=3), (F) cytopins and (G) RNA levels of *Meis1* (*n*=2) of BM 2 and spleen 2 from *MLL*(40-50)^{PTD/WT}, E2A-PBX and *MLL*-AF9 mouse cells treated with 40 μM WDR5-0103 for 5 days. Red arrows indicate differentiated cells. Scale bars represent 6.25 μm. Bar graphs show mean±S.D. (***P*<0.01, paired *t*-test).

MLL(40-50)^{PTD/WT} transformed cells, supporting the fact that the histone methyltransferase activity of *MLL* is crucial for transcriptional activation and downstream leukemogenesis.

In spite of their frequent association with poor prognostic AML, the lack of appropriate leukemia models has significantly hindered the progress in understanding the function of *MLL*-PTD and *MLL* amplification in leukemogenesis.^{1,2} While the relatively long latency and partial penetrance of AML in *MLL*(40-50)^{PTD/WT} mice indicate the requirement of additional events, such as genes co-amplified in the 11q23 amplicon^{8,9} for the development of full-blown leukemia, the study herein provides the first experimental evidence and novel insights into a cooperative transformation mechanism mediated by *MLL*-PTD and *MLL* amplification to induce leukemia in part by epigenetic deregulation of *MLL* downstream targets. Intriguingly, both gain-of-function and loss-of-function *MLL* mutations have been reported in acute leukemia, indicating that the appropriate activity of *MLL* is required for normal development, and its deregulation in either direction can lead to leukemogenic transformation.¹ Instead of being a passive player in *MLL*-PTD leukemia, the study herein demonstrates the pathogenic functions and potential therapeutic value of targeting *MLL* amplification in acute leukemogenesis.

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